

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Mary R. FLACK et al.
Serial No.: 08/379,872 Group: 1205
Filed: January 27, 1995 Examiner: J. Goldberg
For: GOSSYPOL FOR THE TREATMENT OF CANCER

37 CFR § 1.132 DECLARATION

Honorable Commissioner of Patents
and Trademarks
Washington, DC 20231

Sir:

I, Richard Knazek, declare as follows.

1. I am a co-inventor of the subject matter that is disclosed and claimed in the matter of the above-identified application, and I am an expert in the field of the present invention as evidenced by the attached copy of my *curriculum vitae*.

2. I am also a co-author with Y-W. Wu and C. L. Chik of the scientific paper that is attached to the present Declaration [Wu et al., CANCER RESEARCH 49, 3754-3758, July 15, 1989], which paper was cited by the Examiner of the above-identified application, when rejecting claims 1, 3-4 and 13-15 under 35 USC § 103 in an Office Action dated January 24, 1996.

3. The disclosure set forth in the accompanying Wu et al. publication, as it pertains to certain embodiments of the present invention, is a disclosure by myself of subject matter that I (as one of the present co-inventors) am claiming in the above-identified application. In support of this contention, a review of the accompanying reference shows that Figure 2 thereof corresponds to Figure 1 of the present application, Figure 8 corresponds to Figure 2 of the present application, and Tables 2-3 thereof correspond to Tables 1-2 of the present application; thus showing that the accompanying publication corresponds exactly with the present application's disclosure, in at least some aspects.

4. The disclosure that I made in the accompanying Wu et al. publication, occurred within one year of the filing date of U.S. Application Serial No. 07/551,353 (i.e., July 12, 1990), upon which priority for the above-identified application is claimed under 35 USC 120.

5. The accompanying Wu et al. publication names Dr. Y-W. Wu and Dr. C. L. Chik as co-authors with myself, since they worked under my direction and control at the Developmental Endocrinology Branch, NICHD, NIH, in helping to devise certain laboratory studies that were subsequently reported in the accompanying Wu et al. publication (as well as in the above-identified application).

6. Even though Dr. Wu and Dr. Chik are co-authors, with myself, of the accompanying Wu et al. publication, they are not co-inventors of the subject matter that is disclosed and claimed in the above-identified application. This is because they did not take part in the conception of the invention that is being disclosed and claimed in the above-identified application. Again, Dr. Wu (who worked under my direction and control as a "visiting fellow") and Dr. Chik (who worked under my direction and control as a "staff fellow"), mainly helped in devising the assay methods that were used in the laboratory studies that are reported in the accompanying Wu et al. publication.

7. I do not know the current address of either Dr. Wu or Dr. Chik, since we no longer work together at the Developmental Endocrinology Branch of NICHD. However, to the best of my knowledge, I believe that Dr. Wu (a Chinese national) now resides in Beijing, China, having moved there in about 1990. Concerning Dr. Chik, to the best of my knowledge, she now resides in Alberta, Canada, and is employed at a Canadian university. I have no further knowledge concerning either Dr. Wu's or Dr. Chik's current address or whereabouts.

8. While Mary R. Flack and Marcus Reidenberg were not listed as co-authors on the accompanying Wu et al. publication, they are nonetheless co-inventors (with myself) of the subject matter that is disclosed and claimed in the above-identified application. This is because, Mary R. Flack, Marcus Reidenberg and myself were each involved in the conception of the invention that is disclosed and claimed in the above identified application. In this regard, Mary R. Flack and Marcus Reidenberg were not listed as co-authors on the accompanying Wu et al. publication, since they were not directly involved with the laboratory studies reported in the Wu et al. publication, even though they were involved with clinical studies relating to the present invention.

9. The sole co-inventors of the subject matter that is disclosed and claimed in the above-identified application are myself (Richard Knazek), Mary R. Flack and Marcus Reidenberg.

10. I hereby declare that all statements made herein on my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

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States Code and that such willful statements may jeopardize the validity of the application or any patents issued therefrom.

Date: _____

By: _____
Richard Knazek

CURRICULUM VITAE

NAME: Richard Allan Knazek

ADDRESS: 18330 New Cut Road
Mount Airy, Maryland 21771-3702
(301) 607-8229

**DATE AND PLACE
OF BIRTH:** March 23, 1942, Cleveland, Ohio

CITIZENSHIP: United States

MARITAL STATUS: Married, two children

EDUCATION:

1958-1962 B.S. Chemical Engineering, Case Institute of Technology
Cleveland, OH

1962-1963 M.S. Chemical Engineering, Lehigh University, Bethlehem, PA
(Thesis: The Flooding capacities of Pulsed Liquid-liquid
Extraction Columns)

1964-1965 Evening classes in Biology and Biochemistry, University of
Delaware

1965-1969 M.D., Ohio State University College of Medicine, Columbus, OH

1969-1971 Internship and Junior Residency, Duke Hospital, Durham, NC

1973-1974 Clinical Associate, Medical Oncology, NCI/NIH, Bethesda, MD

1980-1981 Clinical Associate, Endocrinology Fellowship Program,
Clinical Center/NIH, Bethesda, MD

BRIEF CHRONOLOGY OF EMPLOYMENT:

1960-1962 (Summers) Junior Engineer in computer programming,
engineering design and engineering development at Standard
Oil of Ohio

1962-1963 Part-time, research on thermo-physical properties of
cryogenic compounds, at Air Products and Chemicals Co.,
Emmaus, PA

1963-1965 Full-time engineering research at the Experimental Station at
E.I. duPont Co. in Wilmington, DE

1966 (Summer) Research on artificial kidney with Dr. Robert Sparks
at Case Institute of Technology, Cleveland, OH

1968-1969 Hematology research in the laboratory of Dr. Charles Mengel,
Ohio State University College of Medicine, Columbus, OH

- 1971-1973 Research Associate (Surgeon, PHS) Laboratory of Biochemistry, Tumor-Physiopathology Section, NCI/NIH, Bethesda, MD
- 1973-1974 Research Associate (Senior Surgeon, PHS) Laboratory of Pathophysiology Section, DCBD, NCI/NIH, Bethesda, MD
- 1974-1985 Senior Investigator (Medical Director, PHS), LPP, NCI/NIH, Bethesda, MD
- 1974-1992 Physician in part-time private practice, self-employed, Rockville, MD
- 1983-1985 Visiting Professor, Institute of Pathology, Lausanne, Switzerland
- 1986-1988 Deputy Branch Chief (Medical Director, PHS), Developmental Endocrinology Branch, NICHD/NIH, Bethesda, MD
- 1988-1990 President and Co-founder, Cellco Advanced Bioreactors, Inc. (Cellco, Inc.), Kensington, MD
- 1990-1994 Senior Vice President, Cellco, Inc., Germantown, MD
- 1994- Clinical Associate Professor, Division of Endocrinology, Diabetes and Clinical Nutrition, The Oregon Health Sciences University School of Medicine, Portland, OR
- 1994- Visiting Scientist, Clinical Gene Therapy Branch, National Center for Human Genome Research, NIH, Bethesda, MD

RESPONSIBILITIES AT CELLCO, INC.:

Co-founded company in 1987.

Obtained first- and second-round venture capital financing.

Developed and commercialized three artificial capillary culture devices to grow large quantities of cells in vitro.

Established commercial corporate alliances and academic research relationships.

Filed device and process patent applications.

Focused technology to produce of lymphocytes and hematopoietic progenitor cells for cellular therapy protocols for cancer, AIDS and genetic disorders.

Directed in-house research and development programs for tumor infiltrating lymphocyte (TIL) therapy of cancer, cytotoxic lymphocyte therapy of AIDS and retroviral transduction of genetically defective lymphocytes.

Coordinated Cellco programs with the physician-sponsored pre-clinical and clinical cellular therapy trials of multiple academic institutions.

HONORS AND OTHER SPECIAL SCIENTIFIC RECOGNITIONS:

- 1967 Landacre Society - "for outstanding student research" - Ohio State University College of Medicine
- 1968 Honorable Mention - SAMA-University of Texas Medical Branch Scientific Forum
- 1969 Phi Delta Epsilon Senior Award - Ohio State University College of Medicine
- 1974 Diplomate in Internal Medicine - Certified by the American Board of Internal Medicine
- 1975 The Inventors Award - National Institutes of Health
- 1976 "Ten Outstanding Young Men" in America - U.S. Jaycees Award
- 1982 Diplomate in Endocrinology and Metabolic Diseases - Certified by the American Board of Internal Medicine
- 1985 The Foreign Duty Service Ribbon - U. S. Public Health Service

SOCIETY MEMBERSHIPS:

Endocrine Society
American Association for Cancer Research
Society for Experimental Biology and Medicine

OFFICE SKILLS:

Word processing, Display Write 3, Word, Word Perfect.

BIBLIOGRAPHY

1. Sparks RE, Lindan O, Blaney TL, Mehall JR and Knazek RA. Removal of waste metabolites from artificial kidney dialyzing fluid. National Heart Institute Grant No. HE 09250, 1966.
2. Knazek RA, Gullino PM, Kohler PO and Dedrick RL. Cell culture on artificial capillaries: An approach to tissue growth in vitro. Science 178:65-67, 1972.
3. Knazek RA, Kohler PO and Gullino PM. Hormone production by cells grown in vitro on artificial capillaries. Exptl. Cell Res. 84:251-259, 1973.
4. Knazek RA and Gullino PM. Artificial capillaries: An approach to tissue growth in vitro. In: Tissue Culture Methods and Applications. Kruse RP and Patterson MD (eds); Academic Press, NY, pp. 321-328, 1973.
5. Knazek, RA. Solid tissue masses formed in vitro from cells cultured on artificial capillaries. Fed. Proc. 33:1978-1981, 1974.
6. Knazek RA and Skyler JS. Production of polypeptide hormones by cells cultured on artificial capillaries. Proceedings of the International Symposium on Growth Hormone; Milan, Italy, 1975. Excerpta Medica; Amsterdam, The Netherlands. Series #381, pp. 177-186, 1976.
7. Knazek RA and Skylar JS. Secretion of human prolactin in vitro. Proceedings of the Society for Exptl. Biol. and Medicine, 151:561-564, 1976.
8. Knazek RA, Lippman ME and Chopra HC. Formation of solid human mammary carcinoma in vitro. J. Natl. Cancer Inst. 58:419-422, 1977.
9. Skyler JS, Rogol AD, Lovenberg W and Knazek RA. Characterization of growth hormone and prolactin produced by human pituitary in culture. Endocrin. 100:283-291, 1977.
10. Lippman MD, Osbourne CK, Young N and Knazek RA. In vitro model systems for the study of hormone dependent human breast cancer. NEJM 296:154-159, 1977.
11. Hillier SG, Knazek RA and Ross GT. Androgenic stimulation of progesterone production by granulosa cells from preantral ovarian follicles: Further in vitro studies using replicate cell cultures. Endocrin. 100:1539-1549, 1977.

12. Knazek RA, Liu SC and Gullino PM. Induction of lactogenic binding sites in the liver of the Snell dwarf mouse. *Endocrinology* 101:50-58, 1977.
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14. Knazek RA. Tissue culture using the artificial capillary system. In: A.I.Ch.E. Symposium Series. Schmeal WR (ed); NY, pp. 120-122, 1970.
15. Ross GT, Hillier SG, Zeleznik AJ and Knazek RA. Gonadotropin and steroid hormone interactions in ovarian follicle growth, atresia and steroid hormone synthesis. In: Proceedings of the VII Meeting of the International Study Group of Steroid Hormones. Research on Steroids. Kloppa A, Learner L, Vandi-Mahen HS and Sciarra F (eds); Rome, Italy. Academic Press, pp. 185-192, 1977.
16. Knazek RA, Liu SC, Graeter RL, Wright PC, Mayer JR, Lewis RH and Gould E. Growth hormone causes rapid induction of lactogenic receptor activity in the Snell dwarf mouse liver. *Endocrinology* 103:1590-1596, 1978.
17. Monaco ME, Lippman ME, Knazek RA and Kidwell WR. Vasopressin stimulation of acetate incorporation into a DMBA-induced rat mammary tumor cell line. *Cancer Res.* 38:4101-4104, 1978.
18. Zeleznik AJ, Hillier SG, Knazek RA, Ross GT and Coon HG. Production of long term steroid-producing granulosa cell culture by cell hybridization. *Endocrinology* 105:156-162, 1979.
19. Gullino PM and Knazek RA. Tissue culture on artificial capillaries. In: Methods in Enzymology. Jakoby W (ed); Academic Press, NY, Vol. LVII, pp. 178-184, 1979.
20. Knazek RA, Liu SC, Bodwin JS and Vonderhaar BK. Requirement of essential fatty acids in the diet for development of the mouse mammary gland. *J. Natl. Cancer Inst.* 64:337-382, 1980.
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26. Hillier SG, Zeleznik AJ, Knazek RA and Ross GT. Hormonal regulator of preovulatory follicular maturation in the rat. *J. Reprod. Fertility* 60:219-229, 1980.
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29. Nissely SP, Knazek RA and Wolff FL. Somatomedin activity in sera of genetically small mice. *Hormone Metab. Res.* 12:156-164, 1980.
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48. Wu Y-W, Chik CL, Albertson BD, Linehan WM and Knazek RA. Inhibitory Effects of Gossypol on Adrenal Function. Acta Endocrinologica 124: 672-678, 1991.
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51. Culver KW, Knazek RA and Blaese RM. High Titer Retroviral Supernatant Produced in an Artificial Capillary Cell Culture System. In preparation.

ABSTRACTS

1. Knazek RA, Kohler PO and Gullino PM. Hormone production by solid tumor masses grown on artificial capillary beds in vitro. Proceedings of the American Association for Cancer Research 14:102, 1973.
2. Knazek RA. Solid tissue masses formed in vitro from cells cultured on artificial capillaries. Symposium on Recent Developments in Research Methods and Instrumentation, NIH, Bethesda, MD, October, 1973.
3. Knazek RA and Gullino PM. In vitro culture of mammary cells on artificial capillaries. VIIIth Meeting on Mammary Cancer in Experimental Animals and Man, Arlie House, VA, 1973.
4. Skyler JS, Rogol AD, Knazek RA and Lovenberg W. Characterization of growth hormone and prolactin produced by human tumors in culture. Eastern Section for American Federation for Clinical Research, 1974.
5. Skyler JS, Rogol AD, Knazek RA and Lovenberg W. Characterization of growth hormone and prolactin produced by human tumors in culture. Proceedings for American Federation for Clinical Research, 1975.
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8. Knazek RA. Formation of solid tissues in vitro by cell culture on artificial capillaries. 82nd National Meeting, American Institute of Chemical Engineers, Atlantic City, NJ, 1976.
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13. Zeleznik, AJ, Hillier, SG, Knazek RA and Ross GT. Tenth Annual Mtg of the Society for the Study of Reproduction (#76), Raven Press, NY, 1977.
14. Nissley SP, Knazek RA and Wolff GL. Demonstration of the growth hormone dependent serum stimulation of DNA synthesis in chick embryo fibroblasts utilizing sera from genetically dwarfed mice. Am. Soc. Biol. Chem. Mtg., Atlanta, GA, 1978.
15. Hillier SG, Zeleznik AJ, Knazek RA, Legallis FY, Rabson AS and Ross GT. Development of a hormonally-responsive transplantable granulosa cell tumor. Endocrine Soc. Mtg., Miami Beach, FL, 1978.
16. Knazek RA, Liu SC, Graeter RL, Wright PC, Mayer JR, Lewis RH, Keller JA and Gould EB. Growth hormone induces prolactin binding sites in the liver of the Snell dwarf mouse. Endocrine Soc. Mtg., Miami Beach, FL, 1978.
17. Knazek RA, Liu SC, Bodwin JS and Vonderhaar BK. Essential fatty acids are required in the diet for development of the mouse mammary gland. XII International Cancer Congress, Buenos Aires, Argentina, 1978.
18. Knazek RA, Liu SC and Vonderhaar BK. Dietary linoleic acid is necessary for development and maintenance for the mouse mammary gland. Am. Assoc. Cancer Res. Mtg., New Orleans, LA, 1979.
19. Dave JR, Knazek RA and Liu SC. Prolactin modifies the fluidity of rat hepatic membranes. AACR Mtg., San Diego, CA, 1979.
20. Knazek RA, Lim MR, Christy RJ, Liu SC and Watson KC. Prolactin suppresses prostaglandin synthesis by rat granulosa cells. International Symposium on Central and Peripheral Regulation of Prolactin Function, Taormina, Italy, October 12-14, 1979.
21. Knazek RA, Christy RJ, Watson KC, Liu SC, Van Gorder PN, Connelly KM and Lim MF. Prolactin modifies FSH-induced prostaglandin synthesis within the rat granulosa cell. Endocrin. Soc. Mtg., Washington, DC, 1980.

22. Dave JR and Knazek RA. Prostaglandin L₂ modifies both prolactin binding capacity and fluidity of mouse liver membranes. Endocr. Soc. Mtg., Washington, DC, 1980.
23. Dave FR and Knazek RA. Changes in prolactin binding capacity and fluidity of mouse liver membranes during pregnancy and lactation. Endocrine Soc. Mtg., Cincinnati, OH, 1981.
24. Knazek RA, Liu SC, Dave JR, Christy RJ and Keller JA. Indomethacin causes a simultaneous decrease of both prolactin binding and fluidity of mouse liver membranes. Endocrine Soc. Mtg., Cincinnati, OH, 1981.
25. Dave JR, Knazek RA and Sears ME. Bradykinin modifies both prolactin binding capacity and fluidity of mouse liver membranes. Am. Assoc. Cancer Res., Washington, DC, 1981.
26. Knazek RA, Liu SC, St. Amand LM, Dave JR and Christy RJ. Cholesterol accentuates the effect of unsaturated fatty acid deficiency on mammary gland development. Am. Assoc. Cancer Res., Washington, DC, 1981.
27. Dave JR and Knazek RA. Changes in the lipid fluidity and prolactin binding capacity of mouse hepatic membranes during postnatal development. FASEB Mtg, Atlanta, GA, 1981.
28. Liu SC and Knazek RA. PG synthesis and binding by growing and regressing rat mammary carcinoma. Intl. Conf. on Prostaglandins and Cancer, Washington, DC, 1981.
29. Dave JR, Rizzo WB, Knazek RA and Schulman JD. Decreased erythrocyte membrane fluidity in adrenoleukodystrophy and adrenomyeloneuropathy. Am. Soc. Human Genetics, Dallas, TX, 1981.
30. Dave JR, Knazek RA and Liu SC. Membrane microviscosity increases in regressing nitroso-methylurea-induced mammary carcinoma. Am. Assoc. Cancer Res., St. Louis, MO, 1982.
31. Rizzo W, Avigan J, Dave J, Knazek R and Schulman JD. In vitro investigations of adrenoleukodystrophy and adrenomyeloneuropathy. Am. Soc. Clin. Res., 1982.
32. Rotondi A and Knazek RA. A rapid method for the measurement of prostaglandin receptors. Fifth International Conference on Prostaglandins, Florence, Italy, 1982.

33. Dave JR, Knazek RA and Brown NV. Prolactin modifies the prostaglandin synthesis, prolactin binding and fluidity of mouse liver membranes. Endocrine Soc. Mtg., San Francisco, CA, 1982.
34. Liu SC and Knazek RA. Prostaglandin binding to NMU-induced rat mammary carcinoma membranes is increased by copper. Endocrine Soc. Mt., San Francisco, CA, 1982.
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36. Karanian J, Rotondi A, Liu SC and Knazek RA. Prolactin modulates the $\text{PGF}_{2\alpha}$ receptor in the rat ovary. Endocrine Soc. Mtg., San Antonio, TX, 1983.
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39. Whitcomb RW, Linehan MW and Knazek RA. Hexacoinic acid decreases ACTH stimulation of cortisol production by human adrenocortical cells in vitro. Endocrine Soc. Meeting, 1987.
40. Wu YW, Chik CL, Albertson BD, Linehan MW and Knazek RA. Effect of gossypol on human and rat adrenal cell membranes. Endocrine Soc. Meeting, Seattle, WA, 1988.

41. Knazek RA, Wu Y-W, Aebersold PM and Rosenberg SA. Culture of Human Tumor Infiltrating Lymphocytes in Hollow Fiber Bioreactors. Am. Assoc. Cancer Res. Mtg, Washington, D.C., 1990.
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43. Knazek RA, Culver KW, Nguyen N and Blaese RM. High Titer Retroviral Vector Production within a Hollow Fiber Cell Culture System for Enhanced T-Lymphocyte Transduction. BioEast 91 Mtg., Washington, D.C. 1991.
44. Knazek RA, Smith S and Epstein DA. Amino Acid Utilization by High Density Culture of Tumor Infiltrating Lymphocytes within in Artificial Capillary System Perfused with a Low-protein Medium. Meeting of the Society for Biological Therapy, Pittsburgh, 1991.
45. Knazek RA, Kidwell WR and Beckner S. Production of Large Numbers of CD8+ Lymphocytes within an Artificial Capillary System, 1992.
46. Flack MR, Pyle RG, Mullens NM, Lorenzo B, Wu Y-W, Knazek RA, Nisula BC, Reidenberg MM. Gossypol in the Treatment of Metastatic Adrenal Cancer. Endocrine Soc. Mtg., 1992.
47. Bresler HS, Burgee KL and Knazek RA. Repetitive Expansion of Large Numbers of CD8+ Lymphocytes within an Artificial Capillary System. Am. Assoc. Immun./Clin. Immun. Soc. Mtg., Denver, 1993.
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49. Rice, HE, Flake AW, Emani VR, Hedrick MH, Knazek RA, Zanjani ED, Harrison MR. Ex vivo Expansion of Hematopoietic Stem Cells from Human Fetal Liver in an Artificial Capillary System. Am. Soc. Hematol. Mtg., St. Louis, Blood 82 Suppl 1: 18A, 1993.

ISSUED PATENTS

Knazek RA, Gullino PM, Dedrick RL and Kidwell WR, "Cell culture on semi-permeable tubular membranes":

United States	3,821,087	05-18-72
United States	3,883,393	02-11-74
Austria	NR 325210	05-18-73
Belgium	799,675	05-17-73
Canada	1,100,067	04-04-73
Denmark	141,409	04-11-73
France	73 17867	05-17-73
Great Britain	1,395,291	05-08-73
West Germany	DE 23 19 120 C2	04-16-73
Italy	N.987281	05-11-73
Japan	975,654	05-18-73
Mexico	1002	04-18-73
Netherlands	73.06659	05-14-73
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Switzerland	564086	05-16-73
Sweden	7306841-3	05-15-73

Knazek RA and Gullino PM. "Cell culture on semi-permeable tubular membranes encased in a porous envelope":

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Knazek RA, Gullino PM and Frankel DS. "Dual circuit woven artificial capillary bundle for cell culture":

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An *in Vitro* and *in Vivo* Study of Antitumor Effects of Gossypol on Human SW-13 Adrenocortical Carcinoma

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ABSTRACT

The present study investigated the *in vitro* and *in vivo* antitumor effects of gossypol on human SW-13 adrenocortical carcinoma cells. *In vitro* gossypol concentrations $\geq 0.5 \mu\text{M}$ reduced the growth rate of the SW-13 cells. Membrane microviscosity was determined by fluorescence polarization of diphenylhexatriene. The membranes of viable SW-13 cells exposed to gossypol became more rigid after a 1-day exposure to gossypol, the polarization constant, P , increasing from 0.229 to 0.352. Gossypol also increased the microviscosities of isolated mitochondrial and microsomal enriched membrane preparations. Tumor was also transplanted into nude mice by s.c. injection of SW-13 cells. A 1-week pretreatment period followed by daily administration of gossypol in which 30 mg gossypol/kg body weight/day was administered via orogastric tube delayed the onset of visible tumor in the subsequent weeks. Five weeks after transplantation, tumor prevalence rate was 95.8% in the control group and 54.5% in the gossypol-treated group. A second experiment, consisting of 12 weeks of gossypol treatment, reduced a preexisting 71% tumor prevalence to 54% while the tumor prevalence increased to 83% in the control group. This was accompanied by a 41.6% mortality in the control group versus 8.3% in the gossypol-treated group. These data suggest that gossypol may provide a beneficial effect in patients with adrenocortical carcinoma.

INTRODUCTION

Gossypol is a naturally occurring component of cottonseed oil (Fig. 1). It has been shown to be an effective antispermato-genic agent in humans and certain experimental animals, including rats, hamsters, and cynomolgus monkeys (1-6). Gossypol has been shown to impair spermatogenesis by a number of mechanisms. Several investigators have recently reported that gossypol is an uncoupler of oxidative phosphorylation (7), that it both stimulates and inhibits respiration (8), and that it also reduces ATP production (9, 10). Other actions of gossypol include its effects on the activity of many membrane-associated enzymes. Gossypol inhibits several enzymes in the electron transport chain, including lactic dehydrogenase X, NAD-isocitrate dehydrogenase, succinyl-CoA synthetase (11, 12), as well as adenylate cyclase (13), phospholipid sensitive calcium-dependent protein kinase (14), and ATPase activity (15). At the membrane level, it also affects the electrochemical properties of lipid membrane and the ordering of membrane lipid matrix (16, 17).

Since gossypol exhibits such a broad spectrum of activities, several investigators have examined gossypol as an antitumor agent: gossypol lengthened the survival of 10-12-week-old C57BL \times DBA/2 F₁ (hereafter called BD2F₁) mice bearing mouse mammary adenocarcinoma 755 (18) and was effective against the TR-ST cells originating from a rat testicular tumor

(19). Gossypol also lengthened the survival of NMRI mice preimplanted with Ehrlich ascites tumor cells (20). Excessive i.p. doses of gossypol given in the latter experiments may have resulted in weight loss and consequent death of the tumor-bearing mice.

We have previously observed that gossypol increased the microviscosity of both mitochondrial- and microsomal-enriched membranes of normal human adrenocortical cells while inhibiting the steroidogenic activity of several adrenocortical enzymes and altering the sensitivity of adrenal cells to adrenocorticotrophic hormone.⁴ These observations and the possible anti-tumor properties led to the hypothesis that there might be a direct and preferential action of gossypol on adrenocortical carcinoma. For this reason, the *in vitro* and *in vivo* effects of gossypol on the SW-13 human adrenocortical carcinoma cells were studied.

MATERIALS AND METHODS

Dulbecco's minimal Eagle's medium, fetal calf serum, glutamine, penicillin, and streptomycin were purchased from Quality Biological, Inc. (Gaithersburg, MD). HBSS⁵ and trypsin-EDTA were obtained from Gibco Laboratories (Grand Island, NY). 1,6-Diphenylhexatriene and tetrahydrofuran were from Aldrich Chemical Co., Inc. (Milwaukee, WI). Gossypol and gossypol acetic acid were gifts from the National Research Institute for Family Planning (Beijing, China). The established line of small cell human adrenocortical carcinoma (SW-13) was purchased from the American Type Culture Collection (Rockville, MD).

In Vitro Gossypol Treatment

Cell Proliferation. SW-13 cells were seeded in a 25-cm² tissue culture flask (Costar, Cambridge, MA) at densities of 1×10^4 cells/5 ml of Dulbecco's minimal Eagle's medium, supplemented with 10% fetal calf serum, 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 units/ml penicillin, and 2 mM glutamine. The cells were grown in a humidified, 37°C incubator with a 5% CO₂/95% air atmosphere. A gossypol stock solution in absolute ethanol was added to the culture medium to yield final concentrations of 0, 0.5, 5, and 50 μM gossypol with a 0.1% final ethanol concentration. After 1, 2, 4, or 6 days of incubation, the culture medium, containing a few floating cells, was removed. Adherent cells were trypsinized (0.1% trypsin, w/v) and counted using a hemocytometer. Cell viability was determined by trypan blue exclusion.

Membrane Microviscosity Measurements. Cultures of the SW-13 cells were rinsed twice with HBSS (pH 7.2), scraped from the culture dishes, centrifuged at $150 \times g$ for 10 min, and homogenized in 0.3 M sucrose in HBSS, using a Teflon-glass homogenizer. After centrifugation at $800 \times g$ for 10 min, the supernatant was removed and centrifuged at $15,000 \times g$ for 20 min, and then at $100,000 \times g$ for 60 min. The $15,000 \times g$ and the $100,000 \times g$ membrane pellets which comprised crude mitochondrial and microsomal preparations, respectively, were homogenized and resuspended in HBSS. The 1,6-diphenylhexatriene was dissolved in tetrahydrofuran at a concentration of 2 mM before being dispersed in HBSS, to provide a final concentration of 2 μM which was then sparged with N₂ for 30 min to remove traces of

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⁵ The abbreviation used is: HBSS, Hanks' balanced salt solution.

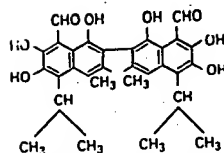


Fig. 1. Chemical structure of gossypol.

tetrahydrofuran before use. The membrane suspensions were diluted to concentrations of 50 μ g protein/1.5 ml. Protein determination was made by a dye binding method using bovine serum albumin as the standard (21). These suspensions were then incubated with an equal volume of the HBSS-1,6-diphenylhexatriene dispersion for 1 h at room temperature. Each membrane sample was subsequently subjected to polarization analysis at 37°C using a Perkin-Elmer MPF-66 fluorescence spectrophotometer (Perkin-Elmer Corporation, Oak Brook, IL). The excitation wavelength was 366 nm, and the emission wavelength was 430 nm. Microviscosity is proportional to the value of fluorescence polarization (P) which was calculated according to the equation:

$$P = \frac{I_v - G \cdot I_H}{I_v + G \cdot I_H}$$

where I_v and I_H are the relative fluorescence intensities measured at an angle of 90 degrees to the incident beam with the emission polarizer in the vertical and horizontal positions, respectively, and $G = I_v/I_H$ measured with the excitation polarizer in the horizontal position.

In Vivo Gossypol Treatment

Nude Mice. Nude mice (Charles River, Kingston, NY) weighing 20–35 g were caged in a temperature-controlled (26–28°C), 12 h/12 h light/dark animal room. A microporous cage bonnet served as an effective protective barrier between the animal and the outside environment. In addition, the room was continuously purged with High Efficiency Particle Attenuator-filtered air. The cages, feeders, and water bottles were designed to make standard mouse chow and water readily available while minimizing the opportunity for the transfer of communicable pathogens.

Transplantation of SW-13 Cells. Forty-nine adult male nude mice weighing 20–24 g were divided into two groups of 24 for control and 25 for gossypol treatment. Gossypol acetic acid was suspended in 75% ethanol for 24 h, then evaporated in vacuum chamber with desiccant, and finally suspended in sterilized 0.25% carboxymethylcellulose carrier. The gossypol-treated group received 30 mg gossypol/kg body weight/day via an orogastric tube. Control mice were fed an equal volume of carrier. Body weights were measured weekly. At the end of the first week of gossypol treatment, 2×10^6 SW-13 cells were injected s.c. on the back of these mice, which continued to receive gossypol or carrier for 5 additional weeks. Tumor surface areas (length \times width, cm^2) were measured daily. After 5 weeks, the animals were decapitated.

Another experiment was designed wherein 48 adult male nude mice weighing 25–35 g were injected s.c. with 2×10^6 SW-13 cells. One month later, the animals were divided into two groups of 24. There were 7 nude mice without visible tumors in each group. The gossypol-treated animals received 30 mg gossypol acetic acid/kg body weight/day whereas control animals were fed an equal volume of carrier. Body weights and tumor sizes (length \times width, cm^2) were measured weekly. During the 12th week of treatment, 5 control animals died. Since it appeared unlikely that the remaining control animals would survive for another week, they were then sacrificed. Autopsies were performed on all animals including those that died during the study period. Internal organs were examined for the presence of gross tumor. Livers and tumors were rinsed with phosphate-buffered saline (pH 7.2) and homogenized in 0.3 M sucrose in phosphate-buffered saline, using a Teflon-glass homogenizer to prepare crude mitochondria and microsomal membranes. The microviscosities of these membranes were then determined as described previously.

Statistical Analysis. Data are expressed as the mean \pm SD unless otherwise indicated. Statistical comparisons were made using an unpaired Student's t test.

RESULTS

Proliferation of SW-13 Cells *In Vitro*

SW-13 cells were seeded in fresh medium. Upon exposure to gossypol at 0.5 μ M they continued to proliferate at almost the same rate as control cells. Gossypol concentrations of 5 and 50 μ M were inhibitory (Fig. 2).

Effect of Gossypol on the Microviscosity of SW-13 Cell Membranes *In Vitro*

Addition of gossypol to suspensions of either microsomal or mitochondrial membranes prepared from SW-13 cells resulted in a rapid, dose-dependent increase in their polarization constant, P . The values of P were then stable during the next 60 minutes (Figs. 3 and 4). Microsomes appear to be more sensitive to gossypol exposure than mitochondria, with 50% inhibitory concentrations of 0.56 μ M for microsomes and 1.4 μ M for mitochondria (Fig. 5).

After *in vitro* exposure of SW-13 cells to 50 μ M gossypol for 2 days, the polarization constant, P , increased from 0.23 ± 0.01 on day 0 to 0.44 ± 0.04 on day 2 whereas P remained unchanged for the control cells incubated without gossypol (Fig. 6).

Effect of Gossypol on SW-13 Tumor Growth *In Vivo*

Study 1. One-Week Pretreatment with Gossypol. Gossypol administered for 6 weeks at a dosage of 30 mg/kg body weight/day by orogastric tube had no effect on body weights. Body weights of control animals at the end of 6 weeks were 24.5 ± 2.5 g compared to 24.6 ± 3.3 g for gossypol-treated animals. After a 1-week pretreatment period with either gossypol or carrier, all animals were inoculated with 2×10^6 SW-13 cells. The subsequent prevalence and average of tumor sizes are shown as a function of time in Table 1. The time required for 50% of the mice to develop tumors were 19 and 30 days for the control and gossypol groups, respectively. The total tumor burden within the control group increased over the 5 weeks subsequent to cell inoculation, reaching a value 4.5 times that of the gossypol-treated group (Fig. 7).

Study 2. Effect of Gossypol on SW-13 Tumor-Bearing Nude Mice. In this experiment, nude mice had been given s.c. injections of SW-13 adrenocortical carcinoma 1 month prior to initiation of the treatment with either gossypol or carrier.

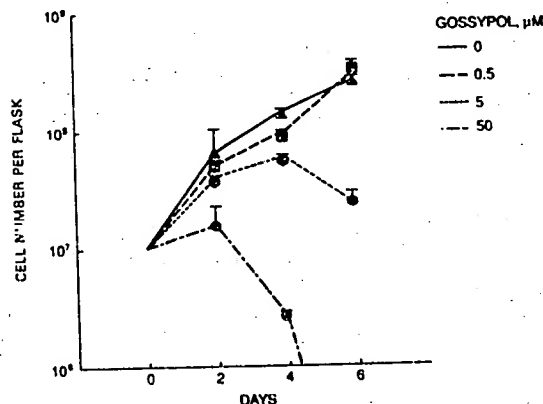
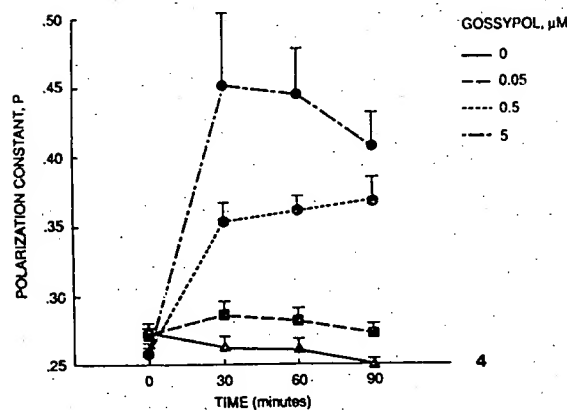
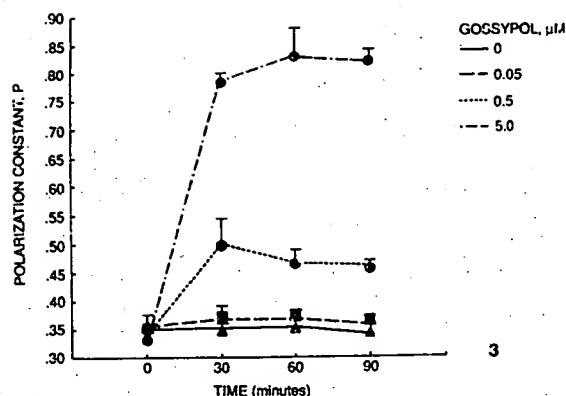


Fig. 2. Proliferation of SW-13 cells during a prolonged exposure to concentrations of 0, 0.5, 5, and 50 μ M gossypol. The SW-13 cells were seeded (1×10^4 cells) into 25-cm² tissue culture flasks in Dulbecco's minimal Eagle's medium supplemented with fetal calf serum (10%), 100 μ g/ml streptomycin, 100 units/ml penicillin, and 2 mM glutamine. Note that exposure to 5 and 50 μ M gossypol inhibited cell proliferation.

ANTITUMOR EFFECT OF GOSSYPOL ON SW-13 ADRENOCORTICAL CARCINOMA



Figs. 3 and 4. The fluorescence polarization constant, P , was determined in SW-13 human adrenocortical carcinoma cells membranes as a function of time after addition of various concentrations of gossypol *in vitro*. Since the value of P is known to be proportional to membrane microviscosity, the data indicate that exposure to gossypol results in an increase in the microviscosity of both the microsomal enriched (Fig. 3) and mitochondrial enriched (Fig. 4) preparations in a dose-related manner.

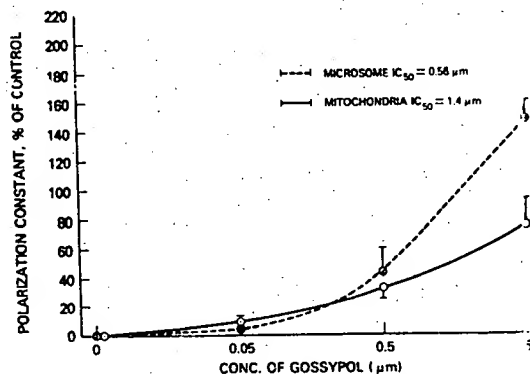


Fig. 5. Gossypol caused increase in the membrane microviscosity of the microsomal and mitochondrial membranes of SW-13 cells *in vitro*. The microsomes appeared to be more sensitive to gossypol exposure than mitochondria, a 50% inhibitory concentration (IC_{50}) of 0.56 μM for microsomes versus 1.4 μM for mitochondria.

During the subsequent 12 weeks of treatment, there were 10 deaths in the control group: 4 had apparent ascites, were jaundiced, and had large intraperitoneal tumors; 2 suffered from hind leg paralysis due to a tumor metastatic to the spinal column; 2 animals had small tumors but both showed significant weight loss; and 2 had demonstrated neither visible tumors nor an obvious cause of death. In contrast, only two deaths were observed in the gossypol-treated group, one of them having

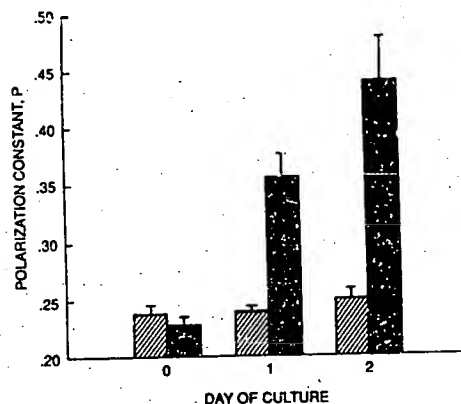


Fig. 6. Incubation of SW-13 cells with 50 μM gossypol caused an increase in the value of the fluorescence polarization constant, P , with time. Gossypol: \square , 0; \blacksquare , 50 μM .

Table 1 Effects of 1 week pretreatment followed by daily administration of gossypol on tumor prevalence and size

Weeks	Control		Gossypol	
	Prevalence of tumor (%)	Av. tumor size (cm^2)	Prevalence of tumor (%)	Av. tumor size (cm^2)
1	12.5	0.08	0	0
2	20.8	0.10	0	0
3	66.7	0.30	33.3	0.08
4	83.3	0.39	34.7	0.09
5	95.8	0.41	54.5	0.11

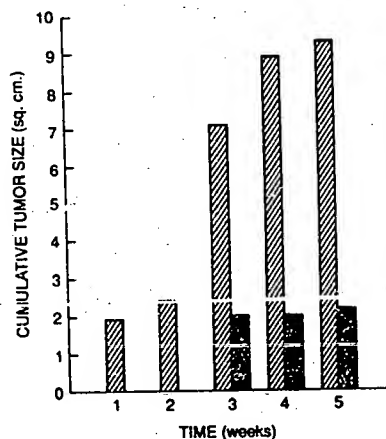


Fig. 7. Gossypol treatment caused a decrease in the cumulative tumor surface area. The tumor sizes (length \times width, cm^2) were measured daily. Tumor-bearing mice received gossypol at a dose of 30 mg/kg/day. The data are presented on a weekly basis. Gossypol (mg/kg/day): \square , 0; \blacksquare , 30.

ascites while the other had no apparent tumor at autopsy. Each treated mouse in the group received a total dose of 81.9 mg gossypol during the 12-week period.

As in the previous study, 12 weeks of gossypol treatment had no significant effect on body weights. At the end of the study period, the body weights in both groups were 32.2 ± 3.8 and 30.9 ± 3.6 g for the control and gossypol-treated groups, respectively. After 12 weeks of treatment, the tumor prevalence had risen from 71 to 83% in the control group, while the gossypol-treated group exhibited a decrease in tumor prevalence from 71% to 54%. This was accompanied by the death of 41.6% of the controls and 8.3% of the gossypol-treated group (Table 2). The mean tumor sizes of the control and the gossypol-treated groups were shown as a function of duration of treat-

ANTITUMOR EFFECT OF GOSSYPOL ON SW-13 ADRENOCORTICAL CARCINOMA

Table 2 Effect of gossypol on tumor prevalence and mortality in mice having preexisting tumors

Week	Control (%)		Gossypol (%)	
	Prevalence of tumor	Total deaths	Prevalence of tumor	Total deaths
0	71	0	72	0
1	75	0	63	0
2	83	0	50	0
3	83	0	54	0
4	83	0	50	0
5	83	0	58	0
6	83	0	58	0
7	83	8.3	58	0
8	83	8.3	58	0
9	83	12.5	54	0
10	83	16.7	54	0
11	83	20.8	54	0
12	83	41.6	54	8.3

Table 3 Effect of gossypol on mean tumor size

Week	Mean tumor size (cm ³) (mean \pm SE)	
	Control	Gossypol
0	0.09 \pm 0.02	0.08 \pm 0.02
1	0.22 \pm 0.05	0.07 \pm 0.02
2	0.28 \pm 0.06	0.11 \pm 0.04 ^a
3	0.35 \pm 0.07	0.15 \pm 0.05 ^a
4	0.50 \pm 0.11	0.20 \pm 0.07 ^a
5	0.66 \pm 0.17	0.28 \pm 0.08 ^a
6	0.87 \pm 0.22	0.32 \pm 0.10 ^a
7	0.97 \pm 0.25 (n = 23)	0.38 \pm 0.12 ^a
8	1.16 \pm 0.33 (n = 22)	0.45 \pm 0.14 ^a
9	1.07 \pm 0.34 (n = 20)	0.50 \pm 0.15 ^a
10	1.14 \pm 0.36 (n = 20)	0.59 \pm 0.18 ^a
11	1.39 \pm 0.41 (n = 19)	0.68 \pm 0.21 ^a
12	0.96 \pm 0.21 (n = 15)	0.81 \pm 0.25 (n = 22)

^a $P < 0.05$, control compared to gossypol treated group; n = 24 unless otherwise indicated.

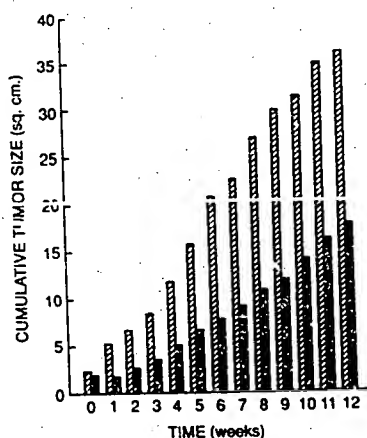


Fig. 8. Effect of gossypol on the cumulative tumor surface areas. The tumor size of each mouse was determined at weekly intervals and expressed as total tumor surface area present in each group. Gossypol (mg/kg/day): \square , 0; \square , 30.

ment in Table 3. The slight decline in the mean tumor size observed towards the end of the study period was due to the fact that the majority of the control mice that died during the study had large tumors.

The total tumor burden of the two groups rose during the treatment period, the controls reaching a value twice that of the gossypol group at the 12th week (Fig. 8). The fluorescent polarization constants of the mitochondrial and microsomal preparations from the livers and tumors of these mice are shown in Table 4. No significant effect of gossypol upon their membrane microviscosity was noted in this *in vivo* study.

Table 4 Polarization constant of liver and tumor membranes obtained from mice in Study 2

	Control		Gossypol	
	Liver	Tumor	Liver	Tumor
Mitochondria	0.25 \pm 0.02	0.26 \pm 0.03	0.26 \pm 0.03	0.22 \pm 0.04
Microsomes	0.26 \pm 0.04	0.27 \pm 0.04	0.26 \pm 0.03	0.26 \pm 0.03

DISCUSSION

Gossypol was shown to suppress the proliferation of SW-13 human adrenocortical carcinoma cells and increase the microviscosity of their membranes. A recent study utilizing artificial membranes indicate that gossypol both binds to phospholipid monolayers with apparent equilibrium dissociation constants ranging from 0.7 to 2 μ M and diminishes the interfacial potential (16). The decreased rate of cell proliferation following gossypol treatment in the present study may, therefore, be a consequence of alterations of phospholipid membranes. Changes in the rigidity of such cell membranes may be a direct effect of gossypol within the membrane or may be secondary to compensatory changes in membrane structure or composition and consequent modification of membrane-associated enzymes.

Pretreatment with gossypol delayed the initial appearance of tumor by 13 days. Furthermore, tumors were detected in 95.8% of the control animals while 54.5% of the gossypol-pretreated group exhibited tumors. The cumulative tumor burden of the control group was approximately 4 times that of the gossypol-treated group. Such findings indicate that gossypol may effectively inhibit early tumor growth.

Established tumors were also shown to respond to gossypol treatment. Tumors eventually appeared in 83% of the control animals but decreased in prevalence from 71% initially to only 54% in the gossypol-treated group. In addition, 41% of the controls died versus only 8.3% of the treated group. Gossypol treatment, therefore, appears to lengthen the survival of SW-13 tumor-bearing male nude mice. Two previous studies have also indicated that gossypol doses of 25–100 μ g/mouse/day or single injections of 0.5 mg/mouse can lengthen survival of NMR mice bearing Ehrlich ascites tumor cells and 10–12-week-old BD2F₁ mice bearing mouse mammary adenocarcinoma (15). However, the gossypol treatment of these previous studies appear to be toxic (18, 20). One major difference between the present and previous studies was the route of administration of gossypol. In the other experiments, gossypol was injected *s.c.*, which in itself may have resulted in higher toxicity. In the present experiment, gossypol was given into animals via an oral gavage tube. A cumulative dose of 81.9 mg gossypol during the 12-week period had no effect on body weight and was accompanied by only one incidental death.

Although gossypol appeared to be safe and effective in delaying growth of SW-13 cells in nude mice, the mechanism of the antitumor action of gossypol remains to be determined. We have previously demonstrated that gossypol inhibits the function of normal human adrenocortical cells, perhaps by increasing the microviscosity of the microsomal and mitochondrial membranes thereby altering the functionality of the membrane-associated steroidogenic enzymes.⁴ The apparent predilection of gossypol to accumulate in the adrenal gland *in vivo* (22) suggested further that this agent might be useful in suppressing the function and growth of adrenocortical carcinoma. The data presented in this paper support this hypothesis, at least with regard to the human adrenocortical carcinoma studied in the nude mouse host. In the *in vitro* study, the membrane micro-

viscosity of the tumor was increased by gossypol treatment, indicating that this generalized membrane effect may be the mechanism by which gossypol could conceivably exert its antitumor effects. However, chronic *in vivo* treatment with gossypol failed to show a significant effect on membrane microviscosity except for a marginal effect on the mitochondrial membranes of gossypol-treated tumors. However, other studies have shown that *in vivo* exposure to agents or environmental conditions that modify membrane microviscosity will result in compensatory changes in the membrane composition to maintain the normal microviscosity. Such may be the case in the present *in vivo* studies which contrast with the *in vitro* experiments in which a marked sensitivity of the membrane microviscosity to gossypol was demonstrated. Compositional studies of the membrane exposed *in vivo* remain to be performed.

The findings of the present study may be of clinical significance in the treatment of adrenocortical carcinoma. Gossypol caused significant regression of preexisting SW-13 tumors and a reduction in overall mortality. The appearance of transplanted SW-13 tumors was also delayed by gossypol pretreatment. These data suggest that gossypol may provide a beneficial effect in patients with adrenocortical carcinoma by decreasing the overall tumor burden and prolonging their duration of survival.

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